IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Short, et al.
Serial No.: 09/997,807
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Art Unit : 1631
Examiner : Michael Borin, Ph.D.

Title: METHOD OF MAKING A PROTEIN POLYMER AND USES OF THE POLYMER

MS RCE
Commissioner for Patents
P.O. Box 1450
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DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

1. I, Nelson Barton, am a co-inventor with Jay Short, Eric Mathur, W. Michael Lafferty and Kevin Chow, on the above-identified patent application.

2. I am an expert in the field of polymer chemistry and was an expert at the time of the invention. I am presently employed as a research scientist at Diversa Corporation, San Diego, CA, assignee of the above-referenced patent application. My resume as documentation of my credentials was attached to a Rule 132 expert declaration submitted in an earlier response.

3. I am familiar with the outstanding issues raised in the last Office Action from the USPTO, and it appears a continuing concern by the Office is whether the specification enabled self-polymerization of conjugated monomers or mostly conjugated monomers. The specification did enable the skilled artisan to make polymers by the self-association of only conjugated monomers or mostly conjugated monomers.

4. One skilled in the art using the teaching of the specification could have made polymers using conjugated monomers with the exemplary protocols described, for example, in Examples 19 and 20, pages 146 to 151, of the disclosure (which are paragraphs [0540] to [0581], of the '681 application). Alternative protocols known to the skilled artisan also could have been used to self-polymerize conjugated monomers of the invention, including using routine modifications of the protocols described in the specification.

5. In further support, that conjugated monomers can self-assemble to form polymers, described herein is a protocol demonstrating self-assembly of exemplary conjugated monomers of the invention (CanA, or SEQ ID NO:2) – monomers conjugated to an enzyme (Green Fluorescent Protein, or GFP) into a polymer. Thus, the specification does enable self-polymerization of only conjugated monomers or mostly conjugated monomers.

6. Studies demonstrated successful self-assembly of the exemplary conjugated monomer of the invention GFP-CanA. The self-assembly of the exemplary CanA is similar to the assembly of other protein polymers, consisting of a relatively slow nucleation step followed by a rapid polymerization step. Therefore, in one embodiment of this invention, in order to improve the rate and efficiency of green fluorescent protein (GFP)-CanA polymer assembly, routine assembly of polymers is accomplished using short canA polymer "primers" to nucleate the GFP-CanA self-assembly reaction. However, as described in the experiments outlined below, the addition of "primers" is not an absolute requirement for the self-assembly of GFP-CanA polymers.

7. Production of Green Fluorescent Protein (GFP)-CanA monomer protein: A 0.5 mL overnight culture (*E. coli* strain carrying plasmid expression cassette for the IPTG-inducible expression of the GFP-CanA fusion protein) grown at 30°C while shaking at 225 RPM in the so-called "terrific broth" (TB), which comprises 1.2% Tryptone, 2.4% yeast extract, 0.4% glycerol, 72mM K2HPO4, and 17mM $KH_2PO_4$) containing 100 ug/ml carbenicillin (called "TB Carb$^{100}$"), was added to 400 mL TB Carb $^{100}$ in a 2 liter baffled flask. The culture was grown under these same conditions until the optical density reached 0.5 $OD_{600}$. At this point, the culture was induced with 0.4 mL 1M IPTG and the growth was allowed to proceed until the optical density increased to 3.2 $OD_{600}$ (approx 8hrs). The culture was then harvested and the whole cells were recovered by centrifugation at @ 6000g for 20min @ 4°C. The cell pellet was resuspended in 10% (w:v) 50 mM Tris pH 7.5, 80mM NaCl, 26 Units/mL DNAse I.

8. Isolation of GFP-CanA monomer protein: The resuspended cell pellet was heat treated at 80°C for 30 minutes resulting both in bacterial cell lysis and denaturation of host cell proteins. The whole cell lysate was centrifuged at 16,773 x g for 30 minutes to produce a clarified

supernatant. A saturated ammonium sulfate solution was added to the heat-treated supernatant to obtain a final 35% saturation. After 18 hours continuous stirring at 4°C, the ammonium sulfate solution was centrifuged at 16,773 x g for 30 minutes to obtain an ammonium sulfate pellet containing the exemplary GFP-Can A monomer of the invention. The ammonium sulfate pellet was resuspended in 5 mL 50 mM Tris-HCl, 80 mM NaCl pH 7.5 and dialyzed in the same solution using a 3 500 MWCO SLIDE-A-LYZER™ (Pierce Biotechnology, Rockford IL) for 6 hours.

9. Self-Assembly of the exemplary GFP-CanA monomer protein of the invention: The dialyzed GFP-CanA monomer solution was added to a Falcon 2059 tube and heated in an 80°C water bath for 5 minutes. Next, $CaCl_2$ and $MgCl_2$ were added, prepared as 1M stock solutions, to give a final concentration of 20 mM $CaCl_2$ and 20 mM $MgCl_2$. The GFP-CanA monomer solution continuously self-assembled into polymer over 12 hours of heating at 80°C. Samples of the polymerization reaction were examined by fluorescence light microscopy using an Olympus model BX51 light microscope fitted with an Olympus MAGNAFIRE SP™ CCD camera and associated image capture software, as illustrated in the attached series of fluorescence light microscope images of self-assembled GFP-CanA polymers of the invention, self-assembled in the absence of CanA "primers".

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted

Date: JUNE 8, 2006

Nelson Barton

# CALIFORNIA STATE SCIENCE FAIR 2004 PROJECT SUMMARY Pyrotex-Optimization of Protein Polymerization





## CALIFORNIA STATE SCIENCE FAIR 2004 PROJECT SUMMARY

Ap2/04

**Name(s)**

**Project Number**

**Project Title**

**Abstract**

**Summary Statement**

**Help Received**

**Ryan M. Short**

## Pyrotex-Optimization of Protein Polymerization

# S0425

**Objectives/Goals**

By recreating the salt conditions, pH, and temperature of the original environment of the organism,
Pyrodictium abyssi, the canA encoded polymerizing protein, will produce the helix forming proteins more
efficiently than with the common laboratory salts magnesium chloride, and calcium chloride used in the
original experimental studies.

**Methods/Materials**

Each cation in the experiments was combined in separate reactions with either calcium, or magnesium.
The results of these reactions were collected and further experiments were performed, based on the results
of which cations were optimal for polymerization. Each reaction occurred at 80°C along with a primer
reactant, consisting of polymerized proteins, to initialize the reaction.

**Results**

What was discovered is that with these salt variables, the nanotubes had over tripled in length compared to
the original controls of the experiment, calcium chloride and magnesium chloride, combined without
additional cations.

**Conclusions/Discussion**

This progress could potentially lead to new breakthroughs in the field of nanotechnology, with countless

applications, from areas such as cardiovascular health, to optical fibers in computers.
The optimization of polymerization conditions of a protein monomer obtained from the isolated
Pyrodictium abyssi microorganism, by recreating the salt conditions, pH, and temperature of the original
deep sea enviornment.
Used laboratory chemicals, centrifuge, and confocal microscope at **Diversa** Corporation, San Diego, CA.,
under the supervision of Dr. Eileen O'Donoghue.